Integrative Properties of Cortical Pyramidal Neurons

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Chapter 5

Attentional load during 5-choice serial reaction time task does not influence PFC c-fos expression in rat

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Abstract

Attention behavior in rodents is widely studied using the 5-choice serial reaction time task (5-CSRTT), which involves accurate behavioral detection of presented visual cues in order to receive a reward. A number of areas of the prefrontal cortex (PFC) are involved in the successful execution of the task. However, studies have mainly relied on lesions of complete brain areas and therefore lack detailed insight on the cellular level into the specific involvement of intracortical networks. Therefore, in the present pilot study we studied layer-specific neuronal activity in a variety of PFC areas by measuring the expression of the neuronal activity-dependent protein c-fos in multiple PFC areas upon 5-CSRTT performance. Rats were tested during conditions with a high or low attention load (high-AL vs. low-AL), which depended on the duration of the stimulus presentation. Rats in the low-AL group showed significantly better performance on all behavioral parameters. However, no layer-specific expression of c-fos could be detected in any of the regions studied. While the small group sizes may have occluded possible effects of attentional load on c-fos expression, the study did produce valuable insights into the behavioral effects of varying the attentional load and the usefulness of c-fos expression as a readout in this behavioral paradigm.

Introduction

The 5-choice serial reaction time task (5-CSRTT) has been widely used to model attention behavior in rodents (Robbins, 2002; Dalley et al., 2004; Bari et al., 2008). During the 5-CSRTT, animals learn to respond to a visual cue presented in one of five possible locations to obtain a food reward. Visual cues are presented briefly and correct responding requires selective attention during the pre-cue phase. Attentional performance in the 5-CSRTT is usually reflected in the accuracy of responding (i.e. number of correct responses/total number of responses, reported as a percentage), the number of missed trials (errors of omission) and reaction times. In addition, responses during the pre-cue phase (premature responses) are used to assess the tendency to impulsivity, while continuous responding after the presentation of the target (perseverative response) is considered an indication of compulsive behavior (Dalley et al., 2004).

Different aspects of 5-CSRTT performance depend on the integrity of various subdivisions of the prefrontal cortex (PFC). Early studies have shown that large lesions of the anterior cingulate (Cg) and prelimbic (PL) cortex result in impaired accuracy of responses, as measured by a reduced ratio of correct responses to the total number of correct and incorrect responses (Muir et al., 1996), suggesting that these areas are involved in attention behavior. However, subsequent studies have shown mixed results regarding the involvement of PFC subdivisions on 5-CSRTT performance. Cg lesions or lesions of the PL and infralimbic cortex (IL) reduced accuracy of responding and also increased the number of omissions, but such an effect could not be observed after lesioning of PL/IL (Passetti et al., 2002). Other studies have reported an increase in omissions also after PL lesions (Chudasama and Muir, 2001; Maddux and Holland, 2011). Lesions of PL/IL also increase the amount of perseverative responses, measured by continued responding after stimulus offset (Passetti et al., 2002), suggesting an involvement of these areas in inhibition of behavior. Together, these results suggest that different PFC areas are involved in specific aspects of attention behavior in the 5-CSRTT.

Direct recordings of PFC neurons have shown how activity is modulated during attention behavior. Using a three choice serial reaction time task, it was shown that neurons in both the PL and the Cg increase their activity during the pre-cue phase. Importantly, the modulation of activity could only be observed during successful trials but not during trials in which the cue was missed (Totah et al., 2009). These results suggest an important involvement of the PL and Cg in the

preparatory attention required for cue detection. In addition, Cg neurons also increase their activity after incorrect trials, suggesting that Cg is involved in both preparatory attention and error monitoring (Totah et al., 2009). A more recent study suggests that the degree of synchrony in the local field potential (LFP) between the Cg and the PL best predicts the behavioral performance in these attention tasks (Totah et al., 2013).

Laminar differentiation is a hallmark of the cortex and the anatomical architecture of the cortex has been widely studied in primary sensory cortex (Bureau et al., 2006b; Oberlaender et al., 2011a; Oberlaender et al., 2011b; Oberlaender et al., 2012). These studies have shown that in different cortical layers, different cell types can be found which vary in physiology (de Kock et al., 2007a; de Kock and Sakmann, 2009b), dendritic morphology (Oberlaender et al., 2011b) and axonal projection profiles (Oberlaender et al., 2011a). However, the sublaminar properties of the PFC have received much less attention. Recent studies have shown that laminar differentiation can be observed in PFC, where the output to subcortical structures shows a small degree of laminar differentiation in rats (Gabbott et al., 2005). For example, projections from the PFC to the ventral striatum can be found more frequently in lower Layer 2/3 (L2/3) and L5, while neurons projecting to the basolateral amygdala are present in L2 and L5 but absent in L3. Finally, projections to the mediodorsal thalamus are restricted to L6 (Gabbott et al., 2005). Distinct layer-specific cell types can be found in the PFC that have unique electrophysiological and morphological properties (Dembrow et al., 2010; van Aerde and Feldmeyer, 2013). Functionally, PFC neurons show differential modulation by, for instance, acetylcholine depending on the layer of origin (Poorthuis et al., 2012) and neurons from different layers also show differences in electrophysiological properties *in vivo* (Boudewijns et al., 2013). Thus, these observations suggest laminar differentiation in the PFC, and therefore layer-specific activity during behavioral task performance could mean differential recruitment of neural networks.

The current study is aimed at elucidating which areas of the PFC show increased activity during conditions of attentional demand. To be able to study the activity in intact animals, a second objective of this study is to investigate whether layer-specific activity can be revealed by measuring the expression of c-fos; an immediately early gene associated with supracritical neural activity (Herdegen and Leah, 1998; Schoenenberger et al., 2009). This method also has the benefit that activity across multiple brain regions can be studied simultaneously within the

same animal. C-fos expression was specifically studied in the Cg, IL, PL, and M1 cortex during conditions of low and high attentional load. This method allows for a direct comparison of the effects of attentional load on activity in different subregions of the PFC and, moreover, can be used to reveal possible layer specific activation of the PFC during attentive behavior.

Methods

Animals:

Eight Sprague Dawley c-fos/lac-z transgenic rats were used (250 - 300 g at the start of the experiment). Animals were housed in pairs in macrolon cages (42.5 × 26.6 × 18.5 cm; l × w × h). Animals were accustomed to a reverse light-cycle regimen (lights on 19:00, lights off 07:00) for one week prior to the start of behavioral training. Simultaneously, a food-restriction diet started that kept animals at 85-90% of their free feeding weight. Water was available *ad libitum* throughout the entire experiment. All behavioral training and testing was performed during the dark phase of the light-dark cycle. All procedures were approved by the Animal Care Committee of the Vrije Universiteit Amsterdam.

Behavioral apparatus:

Behavioral training and testing took place in operant chambers equipped with five holes with infrared detectors and yellow stimulus lights (MEDNPW-5L, Med Associates Inc., St. Albans, VT, USA). Opposite to the nose poke holes was a food dispenser for the delivery of reward pellet. The operant chamber was lit by a white house light located in the wall containing the food dispenser. Operant chambers were placed in sound-attenuated, ventilated boxes. Boxes were controlled and data was collected using MED-PC version 1.17 (Med Associates Inc.). Rats were trained daily, five times per week (Monday-Friday) between 09:00 and 10:00, the total time between first training session and final testing was approximately three months.

Behavioral paradigm:

The first stage of 5-CSRTT acquisition consisted of two sessions of exposure to the operant chamber for 20 minutes, during which a total of 100 pellets were delivered with an average interval of 15 seconds. This stage is designed to allow the animals to associate the sound of the food dispenser with the delivery of a reward. Next, animals proceeded to phase two, in which all the

house light and all cue lights were switched on. A nose poke in any of the five holes resulted in the delivery of a reward and a session was terminated after 100 pellets had been delivered or 30 minutes had passed. Animals were trained in this phase until they reliably obtained 100 pellets. The third phase consisted of trials in which only one cue light was on and rats were rewarded with a food pellet after each poke in the associated hole. Each trial was followed by a 5 second intertrial interval (ITI). Incorrect responses were not punished and the phase was successfully completed when rats reliably earned 100 pellets. Actual acquisition of the 5-CSRTT consisted of illumination of a cue light for 16 seconds until a response was made. Nose pokes in the correct hole within 2 seconds after cue offset were considered correct responses and resulted in the delivery of a food reward. Retrieval of the food reward initiated a new trial, which started the ITI during which all cue lights were off. Nose pokes in holes where the target stimulus was not presented were counted as incorrect responses, whereas the absence of a nose poke was counted as an omission. Both omissions and incorrect responses resulted in a five second time out during which all lights were off. After termination of the time out a new trial was started by illumination of the house light, followed by the next trial. Nose pokes during the ITI (premature) or time outs resulted in a new five seconds time out period (Fig. 1). Animals were trained with a stimulus duration (SD) until they reached 90% accuracy during at least two consecutive trials and then moved on to the next SD. SDs were: 16, 8, 4, 2, 1.5, and 1.0 seconds. After reaching a stable performance in the SD 1.0 condition, rats were tested at SD 0.5 and 0.3 to determine which SD had the largest effect on performance and therefore was expected to create the highest attentional load. On the testing day, one group was tested using SD 5 (low attentional load, low-AL), whereas the other group was tested at SD 0.3 (high attentional load, high-AL). For each cage, one animal was randomly assigned to the low-AL group and the cage-mate to the high-AL group. Rats were perfused 90 minutes after the start of the testing session.

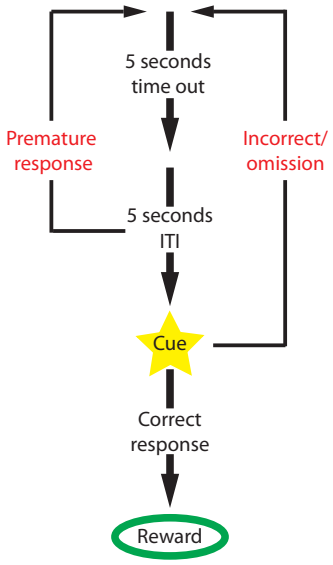

Figure 1: Schematic representation of the behavioral paradigm. *Rats were trained to respond to the appropriate hole after brief light presentation. Trials were initiated every 5 seconds and a correct response was rewarded with a food pellet. Responses before the light onset (premature response) or a response in the wrong hole (incorrect response) or no response after light presentation (omission) resulted in a 5 second time out.*

Perfusion and histology

90 minutes after the start of the test session, rats were deeply anesthetized using pentobarbital (Euthasol, ASTfarma, The Netherlands). When a sufficient depth of anesthesia was assured (determined by the absence of the foot reflex), rats were transcardially perfused with ice-cold NaCl (50 ml) followed by 4% paraformaldehyde (PFA). After perfusion, brains were post-fixed in PFA for one hour and then transferred to PBS.

Before the start of the histological procedures, brains were transferred to 30% sucrose solution until saturated. 40 µm coronal sections were cut using a microtome and sections were stained while free floating. First, sections were rinsed in PBS. Then, sections were treated with 1% H_2O_2, rinsed again and then treated with blocking solution (5% normal goat serum, 2.5% bovine serum albumin, 0.2% Triton-X in PBS). Subsequently, sections were treated with the primary antibodies rabbit-anti-fos (1:5000) and mouse-anti-NeuN (1:1000, Chemicon, Temecula, USA, Chemicon) overnight at 4°C. Sections were incubated in secondary antibodies anti-rabbit alexa 594 (1:400) and anti-mouse alexa 488 (1:400, Molecular Probes, Eugene, USA) for two hours at room temperature and mounted using polyvinyl alcohol mounting medium with DABCO (Sigma-Aldrich, Saint-Louis, USA).

Quantification of c-fos signals:

C-fos labeled neurons were quantified using a confocal microscope (Zeiss LSM 510 meta) at 63x magnification. Images (319x319 µm) were taken of a rostral and caudal section of the cingulate cortex (Cg), prelimbic cortex (PL), infralimbic cortex (IL) and primary motor cortex (M1). For each brain area, layers were identified on the basis of the NeuN staining and pictures were taken of L(ayers) 2/3, 5 and 6. C-fos positive neurons were counted using the cell counting add-on in ImageJ (National Institutes of Health, USA). Images were coded to allow cell counting blind to experimental condition. Neurons were counted as c-fos positive when they displayed a soma-like shape and intensity of the fluorescence signal was higher than three times that of the background. Data are expressed as the number of c-fos positive neurons per 319x319 µm counting area.

Data analysis:

Statistical analyses were performed using Graphpad Instat 3 (GraphPad Software Inc., La Jolla, CA, USA). Due to the low amount of animals per group conservative, non-parametric tests were used: the Kruskal-Wallis test for analysis of variance with Dunn's post hoc test or Mann-Whitney U test two compare two groups. All data are reported as median and interquartile range (IQR). The level of probability for statistically significant effects was set at 0.05.

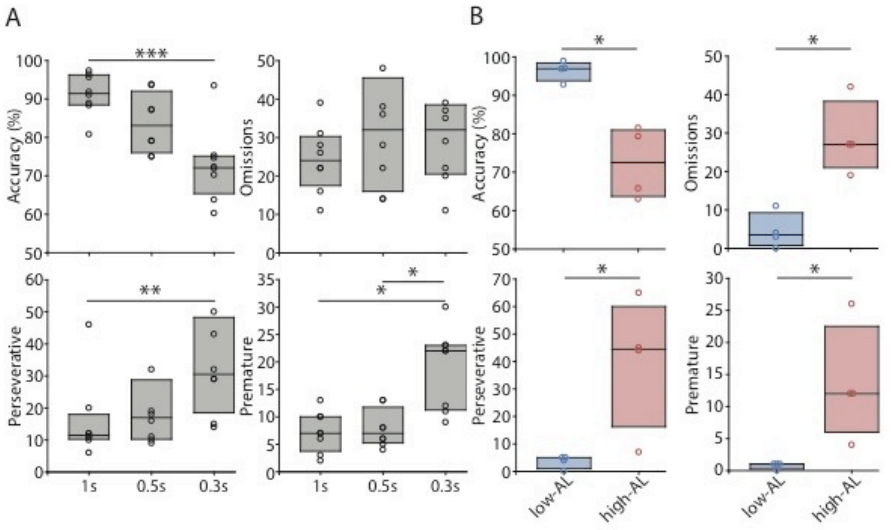

Figure 2: The duration of stimulus presentation affects behavioral performance. A) *Rats were trained to a criterion (80% correct responses) at a stimulus duration (SD) of 1s. Subsequently, SD was reduced to 0.5 and 0.3s. Behavioral performance was significantly reduced at the 0.3s SD.* ***B)*** *On the day of testing, a high attentional load (high-AL, red) group was subjected to a SD of 0.3s, while a low attentional load (low-AL, blue) group was tested at an SD of 5s. Behavioral performance was significantly different between these groups, with high-AL animals showing lower accuracy, more omissions, and more perseverative and premature responding compared to the low-AL group.*

Results

Behavioral data: assessment optimal SD condition

In order to analyze which prefrontal brain areas show activity during 5-CSRTT performance requiring high attentional load, all animals were subjected to tests with SDs of 0.5s and 0.3s on two separate days to investigate the effect of SD on behavioral performance (Fig. 2, Table 1). A main effect of SD on accuracy was observed and post hoc analyses (Dunn's test for multiple comparisons) showed that accuracy was significantly reduced during the SD 0.3s condition versus the SD 1s condition. Reducing the SD did not affect omissions, correct latency time, incorrect latency time, or feeder latency time. The number of perseverative responses increased when SD decreased and post hoc analyses showed that a significant increase was observed when comparing the SD 1s and SD 0.3s conditions but not for other comparisons. Finally, the number of premature responses also increased with decreasing SD. Post hoc analyses revealed that the number of premature responses differed between the SD 1s and SD 0.3s condition

as well as between the SD 0.5s and the SD 0.3s condition. Taken together, since the SD 0.3s condition showed that largest effect on behavioral performance from these experiments, this SD was used for the high attentional load group.

Table 1: *behavioral performance in the 5-CSRTT during conditions of low and high attentional load.*

	SD (1s)	SD (0.5s)	SD (0.3s)	Test-statistic (KW)	*p* value
Accuracy (%)	91.41(7.10)**	83.08(10.77)	72.07(6.18)**	12	.0024
Omissions	24.00(8.25)	32.00(20.50)	32.00(16.00)	1.159	.5602
Latency correct (s)	0.67(0.13)	0.57(0.12)	0.60(0.11)	5.44	.07
Latency incorrect (s)	1.21(0.41)	1.21(0.18)	1.10(0.12)	0.98	.61
Latency feeder (s)	2.30(1.70)	2.17(0.44)	1.99(0.74)	2.44	.30
Perseverative responses	11.50(3.25)*	17.00(11.50)*	30.50(19.25)*	6.213	.0448
Premature responses	7.00(4.75)*	7.00(3.50)	22.00(11.25)*	10.625	.0049

*Data is presented as median (interquartile range); analysis were performed using the Kruskal-Wallis test with Dunn's post test in case of a main effect; * = p < 0.05, ** = p < 0.01; n = 8.*

Behavioral data: high versus low attentional load

To investigate the effect of attentional load on c-fos expression in the frontal cortex, rats were subjected to a test session with low-AL (5s SD) or high-AL (0.3s SD, Fig. 2B, Table 1). Performance in the 5-CSRTT was significantly different between the two groups. Accuracy was reduced significantly in high-AL compared to low-AL rats, whereas the amount of omissions was significantly higher in the high-AL group compared to the low-AL group. The latency to make a correct

response was significantly lower in the high-AL group compared to the low-AL group. No differences were observed in response latencies for incorrect responses and latency to collect the food reward was also similar in both experimental groups. The amount of perseverative responses increased in high-AL compared to low-AL conditions and animals assigned to the high-AL group showed more premature responding compared to rats in the low-AL group.

Table 2: *behavioral performance in the 5-CSRTT during conditions of low and high attentional load.*

	low-AL	**high-AL**	**Test-statistic (U)**	***p* value**
Accuracy (%)	96.92(1.64)*	72.53(14.78)*	0	.0286
Omissions	3.50(3.50)*	27.00(5.75)*	0	.0286
Latency correct (s)	0.53(0.04)*	0.98(0.16)*	0	.03
Latency incorrect (s)	1.00(0.96)	1.26(0.11)	6	.66
Latency feeder (s)	2.22(0.71)	2.19(0.50)	7	.83
Perseverative responses	4.50(2.00)*	44.50 (15.25)*	0	.0268
Premature responses	1.00(0.25)*	12.00(5.50)*	0	.0268

*Data is presented as median (interquartile range); analysis were performed using the Mann-Whitney U test; * = p < 0.05, ** = p < 0.01; n = 8.*

Neuroanatomical data

We next assessed whether the increased attentional load was reflected in changes in c-fos expression in frontal cortical areas between the low-Al and the high-AL group. Therefore, the number of c-fos positive neurons were counted in L2/3, L5, and L6 of the PL, IL, and Cg cortex of rats subjected to either the low-AL or the high-AL condition (Fig. 3). C-fos positive neurons in M1 were counted as a reference, since no differences were expected in locomotor activity between the two groups. We found no significant difference between cell counts in rostral and caudal slices of the frontal cortex, therefore these values were averaged. In the Cg,

no differences were observed between the amount of c-fos positive neurons between the different layers in the low-AL group (Fig. 4A), but in the high-AL group a main effect of layer was found. Post-hoc analyses showed that significantly more c-fos expression was detected in L2/3 neurons compared to L5 neurons. No layer specific c-fos expression was observed in the PL cortex, both in the low-AL and in the high-AL group. In the IL cortex, c-fos expression did not differ between layers in the low-AL, or the high-AL group. Finally, c-fos expression in M1 did not differ between layers in either the low-AL or the high-AL group. Together, these results indicate that with the experimental design used in this study, apart from an apparent increased activation in L2/3 compared to L5 in Cg, no strong layer-specific c-fos expression was observed in the PFC.

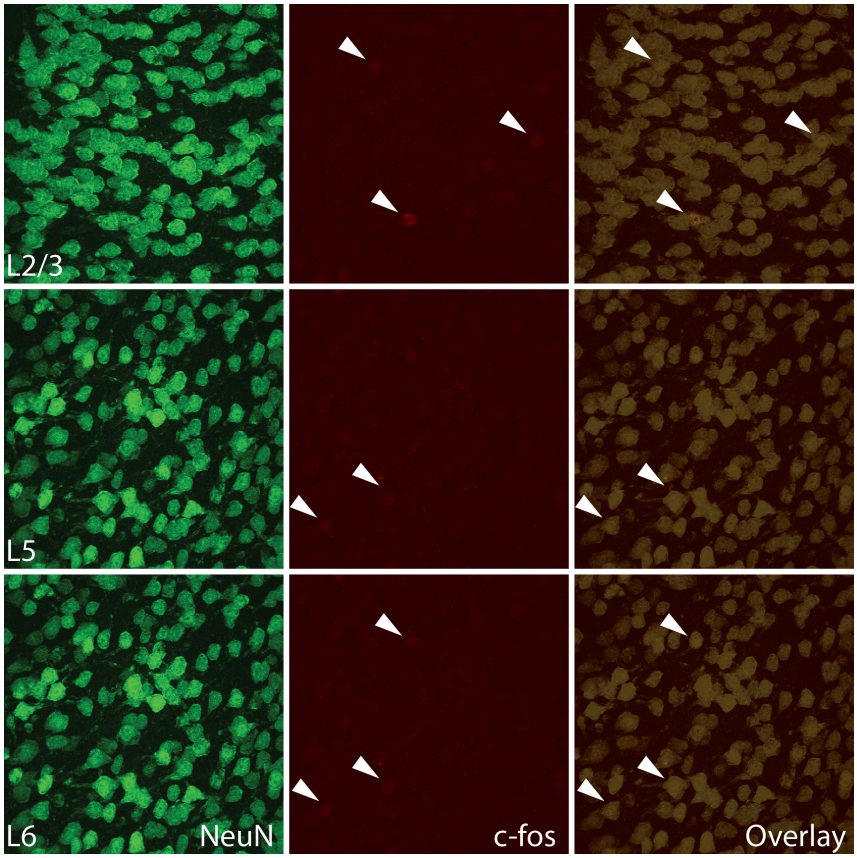

Figure 3: Example of c-fos expression in the PL area of the PFC. *Image of a z-stack of a 319x319 µm area in the PL cortex of the PFC showing NeuN and c-fos expression.*

Table 3: *number of c-fos positive neurons in different PFC areas organized by cortical layer during low and high attentional load.*

	Attentional load	L2/3	L5	L6	Test-statistic (KW)	*p* value
Cg	low-AL	10.80 (4.25)	3.50(3.13)	5.00(4.88)	3.577	.17
	high-AL	12.25(2.88)*	2.00(0.63)*	5.50(4.25)	7.138	.02
PL	low-AL	4.00(2.75)	4.00(3.00)	2.80(1.88)	0.770	.71
	high-AL	7.00(5.50)	3.75(5.13)	3.25(1.38)	3.962	.15
IL	low-AL	8.00(8.82)	8.50(5.00)	2.00(1.00)	3.670	.16
	high-AL	11.25(8.00)	10.25(2.63)	7.00(2.88)	3.228	.21
M1	low-AL	2.50(4.63)	2.75(2.00)	2.50(3.50)	0.090	.96
	high-AL	6.00(5.88)	2.0092.50)	5.25(1.50)	2.586	.30

*Data is presented as median (interquartile range); analysis were performed using the Kruskal-Wallis test with Dunn's post test in case of a main effect; * = $p < 0.05$, ** = $p < 0.01$; n = 4.*

Given the absence of a strong layer-specific effect, data from different layers were poled to test for a main effect of attentional load on c-fos expression in the different brain regions (Fig 4B, Table 4). No effect of attentional load was found in Cg, PL, IL, or M1. Similarly, when data for all PFC areas (Cg, PL, and IL) was averaged, no effect of attentional load was detected (Table 4). In summary, using c-fos as secondary indicator of neuronal activity, we did not observe an effect of high versus low attentional load on PFC neuronal activity during 5-CSRTT performance.

Table 4: *number of c-fos positive neurons in different PFC areas and PFC areas combined.*

Attentional load	low-AL	high-AL	Test-statistic (U)	*p* value
Cg	6.00 (5.50)	7.50(6.88)	6.0	.66
PL	3.25(1.50)	5.00(2.75)	3.5	.23
IL	2.50(3.25)	5.50(4.25)	2.0	.11
M1	9.00(2.75)	5.50(4.30)	4.0	.34
PFC	3.90(1.92)	6.80(2.96)	3.0	.20

*Data is presented as median (interquartile range); analysis were performed using the Mann-Whitney U test; * = p < 0.05, ** = p < 0.01; n = 8.*

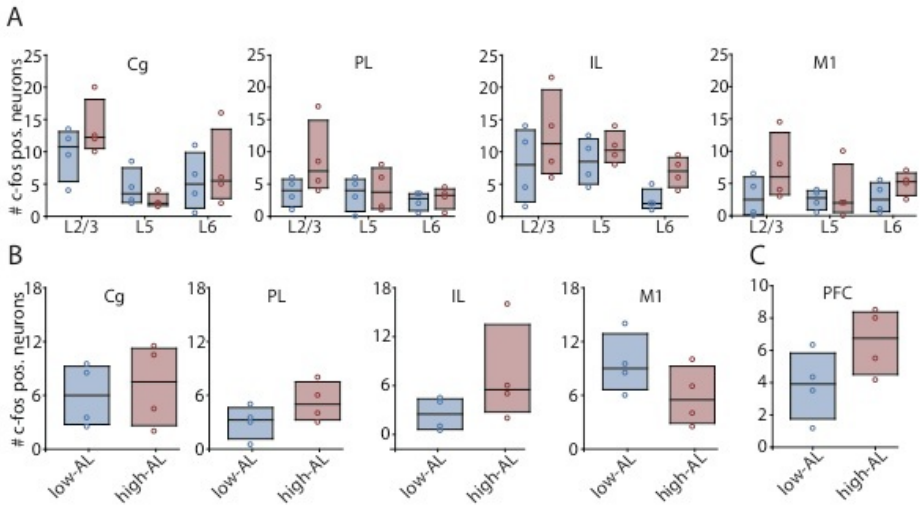

Figure 4: Differences in behavioral performance are not reflected in c-fos expression in PFC areas. A) *C-fos expression in L2/3, L5, and L6 of the Cg, PL, IL, and M1 does not differ between rats subjected to a low-AL or a high-AL load condition.* ***B)*** *When data for layers were pooled, no difference in c-fos expression could be observed between rats subjected to the low-AL and high-AL group for any of the areas investigated.* ***C)*** *When data for the PFC was pooled (Cg, PL, and IL), no difference in c-fos expression could be observed between the low-AL and high-AL group.*

Discussion

The 5-CSRTT is widely used to investigate the involvement of the prefrontal cortex in attention behavior in rats (Muir et al., 1996; Passetti et al., 2002; Dalley et al., 2004; Guillem et al., 2011). However, during the 5-CSRTT a number of cognitive abilities are recruited and each of them have been linked to different structures in the PFC (Dalley et al., 2004). Since most studies linking anatomical regions to behavioral performance have relied on lesions of entire brain areas, little is known about possible layer-specific involvement of neurons in different PFC subregions. Therefore, in this chapter we aimed to determine whether neural activity associated with attentional load during performance of the 5-CSRTT resulted in region-specific expression of c-fos in PFC areas. This approach allowed a direct comparison of different PFC regions and a more detailed analysis of a marker of neural activity in individual layers within these regions during attentional performance.

The results show that behaviorally, as expected, the SD significantly affected behavioral performance in the 5-CSRTT in well-trained rats. Compared to the low-AL group, accuracy was lower and the amount of omissions, perseverative and premature responses were higher in the high-AL group. For rats trained at a SD of 1s, the reduction of the SD to 0.3s increases the difficulty of the 5-CSRTT, which presumably leads to a larger demand on attentional systems. In contrast, the long SD used for the low-AL group greatly reduces the necessity to engage the attention system, as the long presentation of the stimulus makes it difficult to miss. This is especially true considering that the rats tested in the low-AL group were previously trained to perform the task with a much shorter presentation of the stimulus. The reduced difficulty is reflected in the near perfect accuracy and the almost complete lack of omissions. Response latency in the high-AL group was lower compared to the low-AL group during correct responses. While this effect should be interpreted with caution because of the small group size, the response times were consistently lower in all animals in the high-AL group.

Despite the obvious and strong behavioral effects between SD groups, no strong effects of attentional load on c-fos expression could be detected. Similarly, no strong layer-specific activation of neurons could be observed in any of the brain areas investigated. Only in Cg a significant difference was observed in the high-AL group, where more c-fos expression was observed compared to L5. However, taking into account the large number of statistical tests performed, this result should be interpreted with caution as performing a large number of statistical

tests can lead to false positives or type 1 statistical errors. It should also be noted that the employed group sizes in the current described experiments were small. As a result the statistical analyses performed lack the power to show significant effects when differences between groups are small. Therefore, future studies into the layer-specific activity during the 5-CSRTT should be employed using larger group sizes.

In the current study we chose to use c-fos expression as an indicator of activity as it allows for a fast investigation of the involvement of different brain areas during behavioral tasks in intact animals. In retrospect, it could also be argued that c-fos expression is not an optimal method to quantify activity in the PFC during the 5-CSRTT, since effects of varying SD on c-fos expression seem to be small, if present at all. Importantly, performance in the 5-CSRTT involves a number of sequential behaviors including selective attention, reward anticipation, reward collection and error monitoring after incorrect trials, all of which could affect c-fos expression. However, analysis of c-fos expression lacks the temporal resolution to investigate the precise involvement of PFC brain areas in these behaviors (Schoenenberger et al., 2009). In addition, task-specific c-fos expression could have been occluded by neural activity that is not specific to the task. Recently, techniques have been developed that can be used to overcome this issue by using the c-fos promoter to express fluorescent proteins only during a precise time window, while inhibiting the expression during other phases of an experiment (Reijmers et al., 2007; Garner et al., 2012; Liu et al., 2012; Ramirez et al., 2013). These techniques allow a more detailed analysis of neural activity during a task, as non-specific c-fos expression does not interfere with expression that is task-related.

A large subset of neurons shows task-related decreases in activity during the 5-CSRTT (Totah et al., 2009) and these neurons will not express c-fos because the c-fos is increasingly expressed when neurons fire a large number or burst of action potentials (Schoenenberger et al., 2009). In addition, if attentional load is reflected in single spikes the expression of c-fos might not be a suitable readout because of the reliance on a relatively large amount of action potentials. Electrophysiological recordings have the temporal resolution that is required and can be used to measure both increments and decrements in neuronal activity and are therefore more suitable to study the layer-specific activity in the PFC during the 5-CSRTT. Thus, considering the small, non significant effects we find of attentional load on c-fos expression in different areas and layers of PFC, we

conclude that the experimental design does not allow strong conclusions with small numbers of tested animals.

Direct recordings of PFC neurons in a 3-choice attention test have shown that in the Cg and the PL, an increase in neuronal activity is associated with correct responses that cannot be seen during trials where the stimulus was missed (Totah et al., 2009). These results suggest that increased attentional performance is associated with increased activity in these brain areas. The current experimental setup is based on the assumption that areas involved in attention are differentially activated depending on the attentional load of the task. Higher attentional performance would then be associated with increased neural activity in the prefrontal cortex. The low-AL group was chosen as a control group to selectively omit the attention part of the task while keeping all other variables (e.g. motor component of performing the task) similar. However, increased attention performance is usually reflected in a higher accuracy and a reduction in omissions. Since shortening the SD reduced accuracy and increased the amount of omissions, it cannot be concluded that recruitment of brain areas involved in attention was higher in the high-AL group. In addition, even with a long SD there is still an attentional component to the task which could be reflected in neural activity and c-fos expression in the PFC. In future studies it would therefore be crucial to add an additional control group of animals in which c-fos expression in PFC is quantified for naïve animals that remained in their home cage and were not involved in 5-CSRTT.

Together, the experiments described show that reducing the stimulus duration has profound effects on behavioral performance in the 5-CSRTT. Yet, these behavioral changes were not accompanied by differences in activity in PFC areas (as measured by c-fos expression). Apart from the variety of causes that could underlie the lack of differences between the two tested groups, one of the main limitations of the current study is the combination of small differences between groups and small group sizes. Nevertheless, we unarguably show that behavioral performance and quantitative 5-CSRTT parameters are directly correlated to stimulus duration, which could be beneficial for future studies designed to investigate layer specific activation in the PFC during attention behavior.